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MODULATING HIPPOCAMPAL PLASTICITY WITH IN-VIVO BRAIN STIMULATION

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Investigations into the use of transcranial direct current stimulation (tDCS) in relieving symptoms of neurological disorders and enhancing cognitive or motor performance have exhibited promising results. However, the mechanisms by which tDCS impacts brain function remain under scrutiny. We have demonstrated that in vivo tDCS in rats produced a lasting effect on hippocampal synaptic plasticity, as measured using extracellular recordings. Ex vivo preparations of hippocampal slices from rats that have been subjected to tDCS of 0.10 mA or 0.25 mA for 30 minutes followed by 30 minutes of recovery time displayed a robust 2-fold enhancement in long term potentiation (LTP) induction accompanied by a 30% increase in paired pulse facilitation (PPF). The magnitude of the LTP effect was greater with 0.25 mA compared to 0.10 mA stimulations, suggesting a dose-dependent relationship between tDCS intensity and its effect on synaptic plasticity. To test the persistence of these observed effects, animals were stimulated in vivo for 30 min at 0.25 mA then allowed to return to their home cage for 24 hours. Observation of the enhanced LTP induction, but not the enhanced PPF, continued 24 hours following completion of 0.25 mA of tDCS. Addition of the NMDA blocker AP-5 abolished LTP in both control and stimulated rats but maintained the PPF enhancement in stimulated rats. The observation of enhanced LTP and PPF following tDCS demonstrates that non-invasive electrical stimulation is capable of modifying synaptic plasticity.

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1.0 SUMMARY

Investigations into the use of transcranial direct current stimulation (tDCS) in relieving symptoms of neurological disorders and enhancing cognitive or motor performance have exhibited promising results. However, the mechanisms by which tDCS impacts brain function remain under scrutiny. We have demonstrated that in vivo tDCS in rats produced a lasting effect on hippocampal synaptic plasticity, as measured using extracellular recordings. Ex vivo preparations of hippocampal slices from rats that have been subjected to tDCS of 0.10 mA or 0.25 mA for 30 minutes followed by 30 minutes of recovery time displayed a robust 2-fold enhancement in long term potentiation (LTP) induction accompanied by a 30% increase in paired pulse facilitation (PPF). The magnitude of the LTP effect was greater with 0.25 mA compared to 0.10 mA stimulations, suggesting a dose-dependent relationship between tDCS intensity and its effect on synaptic plasticity. To test the persistence of these observed effects, animals were stimulated in vivo for 30 min at 0.25 mA then allowed to return to their home cage for 24 hours. Observation of the enhanced LTP induction, but not the enhanced PPF, continued 24 hours following completion of 0.25 mA of tDCS. Addition of the NMDA blocker AP-5 abolished LTP in both control and stimulated rats but maintained the PPF enhancement in stimulated rats. The observation of enhanced LTP and PPF following tDCS demonstrates that non-invasive electrical stimulation is capable of modifying synaptic plasticity.

2.0 INTRODUCTION

Clinical studies have revealed the potential of transcranial direct current stimulation (tDCS) as a therapeutic tool. tDCS can partially reverse motor impairments induced by stroke (Jo et al., 2009) and Parkinson's (Boggio et al., 2006), and can compensate cognitive deficits induced by Alzheimer's (Ferrucci et al., 2008; Boggio et al., 2009), depression (Fregni et al., 2006; Loo et al., 2012; Brunoni et al., 2014), schizophrenia (Goder et al., 2013) and post-traumatic syndrome disorder (PTSD) (Saunders et al., 2014). In addition to these clinical benefits, tDCS use in healthy subjects has been observed to improve declarative and working memory (Marshall et al., 2004; Fregni et al., 2005; Jeon and Han, 2012; Hoy et al., 2013), and other cognitive functions (Fiori et al., 2011; Chrysikou et al., 2013; Martin et al., 2014).

There is an immense volume of work documenting the effects of various forms of electrical stimulation on neuronal activity. In 1956, it was discovered that weak current stimulation in crayfish resulted in sub-threshold changes in membrane potential inducing either neuronal hyperpolarization or depolarization thus translating to either inhibition or excitation, respectively, depending on the polarity of applied current (Terzuolo and Bullock, 1956). It was subsequently demonstrated that polarizing current applied to the exposed cortex of an anesthetized rat for at least 5 min produced enhancement in evoked response and spontaneous activity that persisted for at least 3 hours after cessation of polarizing current stimulation (Bindman et al., 1962). Follow up studies in humans later indicated that tDCS lasting at least 5 min applied to the motor cortex induced a significant increase of approximately 150% in motor evoked potential which can be readily measured (Nitsche and Paulus, 2000) but that such enhancement can only last up to 90 min after the end of stimulation (Nitsche and Paulus, 2001).

Recent work using rats subjected to in vivo anodal tDCS corroborates human studies, revealing increased cortical excitability and improvements in working memory, skill learning and motor coordination as assessed using a variety of behavioral tests (Dockery et al., 2011; Binder et al., 2014; Romero Lauro et al., 2014). Also consistent with human studies, anodal tDCS has been demonstrated to possess therapeutic potential in rat models of Alzheimer's (Yu et al., 2014) and stroke (Jiang et al., 2012). However, the cellular mechanism by which anodal tDCS exerts its effects remains elusive. Based on past studies on the enhancement of learning and memory in both human and animals, there is a general consensus that anodal tDCS could enhance synaptic plasticity, especially LTP. In vivo application of tDCS in healthy human subjects produced a short-lasting plasticity in the motor cortex as measured by motor-evoked potentials (Fricke et al., 2011). Similarly, in vivo stimulation in rabbits suggested that tDCS can modify synapses at pre-synaptic sites that are essential for associative learning (Marquez-Ruiz et al., 2012). In vitro exposures of brain slices to anodal current stimulation enhanced synaptic plasticity in mouse motor cortex (Fritsch et al., 2010) and in CA1 neurons of rat hippocampus (Ranieri et al., 2012). Furthermore, in vitro current stimulation applied directly to rat hippocampal slices has been shown to alter amplitude and frequency of gamma oscillations, mathematically predicted to be induced by changes in synaptic function (Reato et al., 2014).

There is limited data available on the direct effects of in vivo tDCS on cellular LTP. Here, we show that in vivo application of anodal tDCS in rats (0.25 or 0.10 mA for 30 min) induced a significant enhancement in LTP and PPF in the Schaffer collateral-CA1 synapse of the

hippocampus. The enhanced effect on LTP in hippocampal slices was dependent on tDCS intensity, and persisted for at least 24 hours following completion of tDCS. Additionally, we show that the observed tDCS-enhanced LTP at the Schaffer collateral – CA1 pathway is dependent on NMDA receptors whereas tDCS-enhanced PPF is independent of NMDA receptors.

3.0 METHODS

Animal handling

All rats were maintained according to National Institute of Environmental Health Sciences and Wright Patterson Air Force Base (WPAFB) Institutional Animal Care and Use Committee guidelines. The study protocol was reviewed and approved in compliance with the Animal Welfare Act and with all applicable Federal regulations governing the protection of animals in research.

All animals (7 week old male Sprague Dawley rats) were purchased from Charles River and received a 10 day acclimation period upon arrival to WPAFB facilities prior to surgical implantation of an electrode. A total of 34 rats were used for this study. Rats were monitored for one week to assess recovery before being randomly selected for sham or tDCS treatment, tDCS stimulation and electrophysiological procedures.

Surgical implantation of cranial electrode

Animals were anesthetized with isoflurane (Shopmedvet) using 5% induction followed by 2-3% isoflurane to maintain anesthetic depth. The head was stabilized using stereotax for the procedure. Briefly, a rostral-caudal incision was made to reveal the skull and a lateral incision was made at the shoulders for exit of electrode wire. A head electrode of .25 cm2 (ValuTrode, Avelgaard Manufacturing Co., 1.25 inch diameter circular electrode cut to 5mm x 5mm) was applied to the skull with the center of the electrode resting on the midline 2.5 mm caudal to bregma. The insulated electrode wire was tunneled subcutaneously and exited the lateral incision. A c-clamp was then placed on the skull. C&B Metabond Adhesive Luting Cement (Parkell) was then applied to bond the electrode to skull. Acrylic dental cement (Sigma) was then applied to fill space between electrode and clamp to secure electrode in place. Incisions were then closed around cement and wire by suturing. A minimum of 7 days recovery was permitted prior to tDCS treatment.

tDCS Treatment

Five minutes prior to stimulation, animals were removed from homecage, weighed and brought to the experimental room. The head electrode was inserted into experimental wires and a reference electrode (8.04 cm2, ValuTrode, Axelgaard Manufacturing Co.) was placed between the shoulders with Signagel electrode gel (Parker Laboratories) as the conducting medium. The animal was then briefly restrained manually to allow for the reference electrode to be secured via Petflex cohesive bandage tape (Shopmedvet). Once the electrodes were in place, the animal was placed into a novel environment made of plexiglass, containing two novel objects for

exploration. Animals were allowed to freely move throughout stimulation and were monitored via Ethovision software. tDCS was then applied using a constant current stimulator (Magstim DC-stimulator, Neuroconn) for 30 minutes. The animals that received sham stimulation received the same treatment; however wires were left unhooked from stimulation device. Following stimulation, the animals were returned to their homecage until time of euthanasia and brain slice preparation (30 minutes or 24hrs post stimulation).

Brain slice preparation

Rats were euthanized using rapid decapitation. Brain and brain slices were kept viable by keeping in ice cold artificial cerebrospinal fluid (ACSF) that was kept continuously oxygenated (95/5 O2/CO2). ACSF consists of (in mM): 124 NaCl, 3 KCl, 1.25 KH2PO4, 10 D-Glucose, 1 MgSO4, 36 NaHCO3, and 2 CaCl2 (pH ~7.4). Cerebellum and approximately 1 cm of frontal cortex were removed and the remaining brain was sectioned at 350 µm thick using a vibratome (VT1000S Leica Microsystems or OTS-4000 FHC Inc) in the transverse plane, at 20-30□ laterally off the horizontal axis. Brain slices were maintained in oxygenated ACSF and allowed to recover for at least 60 min prior to recording. A new batch of ACSF was prepared each morning of experimentation and continuously oxygenated with 95% O2 and 5% CO2.

One hippocampal slice was placed onto the pre-coated MED64 probe, using small weights to anchor the slices down. The probe containing the brain slice was then assembled with the MED64 system, as specified in the MED64 instruction manual. A perfusion cap was used to circulate fresh oxygenated ACSF into the probe and prevent the slices from drying. The ACSF solution and oxygen entering the probe chamber were maintained at 32-34°C. Flow rates were maintained at approximately 0.5 to 1.0 mL/min while ensuring a liquid-air interphase. Humidified oxygen entered the probe at about 0.3-0.5 L/min.

Electrophysiology recording

All electrophysiology recordings were blinded experiments, in which the exposure condition of the rat (tDCS or Sham) was not identified until the completion of recordings from all rats in the same cohort. A cohort is one group of rats of the same age that has undergone electrode placement surgery on the same day.

All electrophysiology data were obtained using AlphaMed's MED64 (Automate, Berkeley, CA), an extracellular recording system containing 64 planar microelectrodes arranged in an 8x8 array. Data acquisition and stimulation protocols were performed using Mobius software (Automate, Berkeley, CA). A stimulating current of 10-100 µA was applied to the Schaffer collateral region of the hippocampus to obtain an input/output relationship curve (Fig. A-1). Evoked field potentials in the form of field excitatory post-synaptic potentials (fEPSP) and population spikes were obtained in the CA1 region of the hippocampus (Fig. A-1) and recorded every 6 seconds. Using the input/output relationship curve, we determine the size of the stimulating current that resulted in half of the maximal output response. Typically, a stimulating size of 30-50 µA induced the half-maximal response and thus was used in our experiments. Baseline recording was obtained for each slice for at least 30 min prior to LTP measurements. LTP of CA1 neurons was induced by delivering 3 trains of theta burst stimulation (TBS), consisting of 10 repeats of 4

high frequency stimulation (100Hz) every 200 ms to the Schaffer collateral regions. Evoked responses or field potentials (fEPSPs and population spikes) were monitored at 6 second intervals for at least 30 minutes following LTP induction. Percent potentiation was calculated by computing the percent difference in population spike amplitude or fEPSP slope at either 30 or 60 min following LTP induction by TBS from baseline. Averages of 5 data points were calculated to obtain baseline and LTP values. Paired pulse facilitation (PPF) was obtained by delivering two consecutive stimuli at 50 µA that are 40 ms apart. To ensure that facilitation was not present at 24 hrs, additional recordings were made with paired stimuli at 30 and 40 µA. Responses mediated by the AMPA and kainate receptors were blocked using 30 µM DNQX (6,7-Dinitroquinoxaline-2,3(1H,4H)-dione, Sigma). NMDA receptors were blocked using 50 µM AP-5 (D(−)-2-Amino-5-phosphonopentanoic acid, Sigma).

Data analysis

Field excitatory post-synaptic potentials (fEPSPs) were calculated by fitting a line to the initial rise of the evoked response and calculating the slope in the CA1 region. Amplitudes of population spikes were also calculated. Slope and amplitude calculations were performed using Mobius software (Automate, Berkeley, CA). Quantitation of LTP was obtained by averaging 5 data points at the indicated times (just prior to LTP induction by TBS and at 30 or 60 min following TBS). Percent LTP or percent potentiation refers to the slope or amplitude of fEPSP at either 30 or 60 min after TBS minus the slope or amplitude of baseline fEPSP prior to TBS divided by the slope or amplitude of baseline fEPSP values. Normalized fEPSP data refers to the slope or amplitude of fEPSP divided by the average slope or amplitude of all fEPSP points prior to TBS. Quantitation of PPF was obtained by dividing the slope or amplitude of the fEPSP response due to the second stimulus divided by the slope or amplitude of the fEPSP response due to the first stimulus to obtain the PPF ratio. Data from multiple MED64 microelectrodes within the CA1 region of a hippocampal slice were averaged together to obtain the response from that particular slice. Typically, 1-2 hippocampal slices per rat were used and n values are indicated as the number of rats followed by the number of slices used. For our PPF data analysis, multiple stimulations at distinct locations in one slice were performed and various microelectrodes within the CA1 region were recorded and counted as the sample size in the statistical analysis of this data.

Data are represented as means with the standard error of the mean (SEM) and were statistically compared using an unpaired, two-tailed t-test. A calculated P of less than 0.05 is considered significantly different. All quantitation and statistical analysis as well as graphs were generated using Microsoft Excel and Sigmaplot v.12.5.

4.0 RESULTS

In vivo anodal tDCS in rats enhanced LTP in acutely prepared hippocampal slices

To determine the optimum stimulation intensity for LTP experiments, we applied multiple stimulating currents to the Schaffer collateral region of rat hippocampal slices ranging from 10 µA to 100 µA in intensity (Fig. A-1a). We found that a current of 50 µA consistently induced half-maximal response and thus a 50 µA-current was used as stimulus for our

electrophysiological experiments. There was no obvious effect on the size and shapes of the evoked response in the hippocampus of control or stimulated rats (Fig. A-1b). Furthermore, tDCS did not induce changes in the frequency of spontaneous activity in CA1 region of the hippocampus resulting from tDCS (Fig. A-1c).

LTP was induced using 3 trains of theta burst stimulation (TBS). We found that there was a significant increase in the degree of LTP in rats that were subjected to tDCS compared to control rats (Fig. A-2). By calculating the initial slope of the field potentials at 30 minutes following LTP induction using TBS, there was a 63.7±6% potentiation in control rats (Sham) whereas a 129.6±16% potentiation in stimulated rats (tDCS) (Fig. A-2a,c). At 60 minutes, the difference is further enhanced, resulting in 52.9±5% potentiation in control rats and 135.2±14% in stimulated rats (Fig. A-2a,d). Statistical analysis using unpaired, 2-tailed t-test yielded P values of 0.01 and 0.002 for the 30- and 60-min slope data, respectively. Amplitudes of field potentials were also calculated, yielding a 42.8±3 % and 93.5±9% potentiation in control and tDCS-treated rats, respectively at the 30-min time point, and 42.3±4% and 92.3±9% potentiation in control and tDCS-treated rats, respectively at the 60-min time point (Fig. A-2b,e,f). P values were <0.001 for both the 30- and 60-min amplitude data. The differences observed at 30 min were always observed at 60 min and were of a greater magnitude (Fig. A-2d). Therefore, in subsequent experiments, the percent LTP potentiation of evoked responses were calculated only at the 30 min time point following LTP induction.

Anodal tDCS in rats enhanced PPF in acutely prepared hippocampal slices

PPF measurements were obtained by delivering two 50 μA stimuli that are 40 ms apart to the Schaffer collateral region of the hippocampus and evoked responses from CA1 region were recorded. We found that there was significantly greater PPF in stimulated rats compared with control rats (Fig. A-2e). Rats subjected to 30 min of tDCS (250 μA) followed by 30 min recovery time displayed PPF ratio of 1.5±0.04 compared to 1.1±0.09 of control rats when slope measurements were used (P = 0.003). Similarly, PPF ratio values based on amplitude calculations were 1.5±0.1 and 2.2±0.07 for control and tDCS-treated rats, respectively (P = 0.005).

Dependence of synaptic plasticity on tDCS intensity

The enhancing effect on LTP and PPF was still observed, albeit to a smaller extent, when tDCS intensity was decreased from 250 μA to 100 μA (Fig. A-3a,d). Hippocampal slices obtained from rats treated with 30 min of 100 μA tDCS followed by 30 min recovery time resulted in a percent LTP of 118.5±16% compared to 83±7% from control rats (Sham) as measured by calculating the slopes of field potentials (P = 0.01) (Fig. A-3b). Amplitude measurements were 54±7% LTP for control rats and 88±17% for stimulated rats (P = 0.04). Furthermore, slopes of evoked response in hippocampal slices from tDCS-treated rats still displayed greater PPF ratio (1.7±0.1) compared to sham-treated rats (1.3±0.05) (Fig. A-3d; P = 0.002) . Slope values for PPF ratio were 2.4±0.06 and 1.6±0.2 for tDCS- and sham-treated rats, respectively (Fig. A-3d; P = 0.006).

Lasting effects of tDCS on synaptic plasticity

To determine whether the effects to tDCS were persistent, animals received in vivo tDCS stimulation for 30 min then were returned to their home cage for 24 hours. We observed that the effect of tDCS on LTP was still maintained 24 hours post-tDCS (Fig. A-4). Using field potential slope values, the Sham group experienced an average LTP of 76±9% whereas the tDCS group experienced an average LTP of 154±35 % ($P = 0.03$, $N = 7$ rats, 8 slices). Similarly, using amplitude values, the Sham group experienced an average LTP of 47±8% whereas the tDCS group experienced an average LTP of 146±50% ($P = 0.02$). Unlike the data obtained from rats 30 minutes post-tDCS, data obtained from rats 24 hours post-tDCS did not reveal significant changes in PPF. We stimulated hippocampal slices with two consecutive stimuli that are 40 ms apart, at 3 different intensities (30, 40, and 50 µA) and did not detect any significant effects on the PPF ratio ($P > 0.1$).

Dependence of tDCS–mediated plasticity effects on NMDA receptors

The observed field potentials from CA1 neurons were predominantly mediated by ionotropic glutamate receptors since the evoked response was quickly abolished by the perfusion of 30 µM DNQX and 50 µM AP-5 in hippocampal slices from both control and tDCS rats (Fig. A-5). Application of 50 µM AP-5 only minimally reduced the evoked response but prevented LTP induction in both sham and tDCS-treated rats (Fig. A-5A-C). However the enhanced effect on PPF was still observed in the presence of AP-5 (Fig. A-5D). This suggests that the tDCS-induced increase in PPF is not NMDA-dependent, as blockade of NMDA receptor still induced PPF ratios of 1.4±0.1 in tDCS-treated rats compared to a PPF ratio of 1.1±0.08 in control rats when amplitude values were used ($P = 0.02$) and PPF ratios of 2.7±0.5 and 1.6±0.2 in stimulated and control rats respectively when slope values were used ($P = 0.03$). Our data is consistent with the previously established principles claiming that calcium accumulation in the pre-synaptic terminal is critical in establishing PPF. We believe this is the first account of in vivo tDCS enhancing plasticity of neurons at both the pre- and post-synaptic sites of rat hippocampus.

5.0 DISCUSSION

We have demonstrated that in vivo tDCS in rats can enhance LTP and PPF, two distinct types of synaptic plasticity in the rat hippocampus. Reduction of tDCS intensity from 200 µA to 100 µA decreased the LTP enhancement to approximately 1.4 fold, supporting the possibility of dose-dependent effects. This is consistent with earlier findings in which modulation of cortical excitability was dependent on current stimulation intensity (Bastani and Jaberzadeh, 2013; Murray et al., 2014). Of interest, the effect on PPF appears to be all-or-none, enhancing PPF by about 30-50% at both the high and low tDCS intensities. Although the increase in PPF can no longer be detected at 24 hours following completion of tDCS, the LTP enhancement still persists suggesting the possibility that tDCS-mediated enhancements of LTP and PPF occur through distinct mechanisms. Our data further suggest that tDCS-induced increase in LTP is NMDA-dependent which is consistent with the previously established principles that post-synaptic NMDA receptors play a critical role in LTP at the CA1 region of the hippocampus, as well as with other studies demonstrating that tDCS-induced effects can be blocked by NMDA blockers

(Nitsche et al., 2004). We did not observe any significant changes in the size and shape of the evoked responses or a change in frequency of spontaneous activity in the CA1 region of rat hippocampus. Components of evoked responses in the CA1 region were mediated by primarily ionotropic glutamate receptors, as perfusion of the kainate and AMPA blocker DNQX, combined with the NMDA blocker AP-5, blocked all excitatory evoked responses from both control and stimulated rats. Our data therefore rule out the mechanistic possibility of tDCS producing effects that recruit or enhance other non-glutamatergic synapses in the measured CA1 region of the hippocampus.

A rigorously studied form of synaptic plasticity is long term potentiation (LTP), discovered first in the perforant path of an anesthetized rabbit (Bliss and Lomo, 1973). Further work in brain slices revealed that the CA1 pyramidal cells of rat hippocampus consistently undergo LTP upon high frequency stimulation (Dunwiddie and Lynch, 1978). Since then, LTP has been studied extensively not only in the hippocampus but also in other brain regions, and it has been widely accepted as the molecular basis for learning and memory (Izquierdo, 1994; Neves et al., 2008). Although the complete molecular mechanisms of LTP remain under investigation, many of the key players have been identified (Baudry, 2001). Data suggest that high frequency stimulation induced recruitments of post-synaptic ionotropic glutamate receptors (AMPA and NMDA receptors) onto the post-synaptic cell as well as gene expression changes (Baudry, 2001). Although there are different types of LTP, a robust and well-studied form is the NMDA-dependent LTP that persists in the Schaffer collateral-CA1 region of the hippocampus. NMDA-dependent LTP has been shown to be essential for learning and memory as administration of NMDA receptor blocker AP-5 prevented LTP induction and impaired learning (Izquierdo, 1994; Gruart and Delgado-García, 2007; Caroni et al., 2012).

The tDCS-induced enhancement of LTP observed in this study is consistent with previous findings of improved cognitive functions in diseased and healthy subjects resulting from in vivo non-invasive stimulations (Bastani and Jaberzadeh, 2013; Heise et al., 2014). Furthermore, our data is also in agreement with earlier work that in vitro current stimulation directly on brain slices resulted in immediate augmentation of NMDA-dependent LTP in the Schaffer collateral-CA1 pathway of rat hippocampus (Ranieri et al., 2012) and the NMDA and BDNF-dependent LTP in the mouse motor cortex (Fritsch et al., 2010). We measured LTP only on the Schaffer collateral-CA1 pathway of the hippocampus that is mainly NMDA -dependent. Therefore, we questioned whether the observed effect on LTP was due to increased recruitment of other glutamate receptors to the post-synaptic site to generate other types of LTP that is not dependent on NMDA receptors. However, this idea was ruled out by the fact that perfusion of AP-5 blocks LTP in both control and tDCS-treated rats. Although this does not eliminate the possibility that tDCS can also affect other non-NMDA forms of LTP, it strengthens the hypothesis that NMDA receptor is an essential target whereby tDCS exerts its effect, at least in the Schaffer collateral-CA1 pathway.

Paired pulse facilitation (PPF) is another form of synaptic plasticity but in contrast to LTP, it is short-lived and mediated pre-synaptically, resulting from accumulation of calcium ions due to two stimulating pulses delivered within a short inter-pulse duration. PPF is observed when two consecutive stimuli are delivered, within tens of milliseconds of each other, resulting in a potentiated post-synaptic response elicited by the second stimulus. The prevailing mechanistic

explanation of PPF is the transient build-up of calcium ions during two consecutive stimuli (Katz and Miledi, 1968; Thomson, 2000). The second stimulus produces an unusually larger calcium pool, which will subsequently trigger greater release of neurotransmitter molecules (Katz and Miledi, 1968; Thomson, 2000). This is unlike LTP where the likely mechanism involves mainly post-synaptic events such as the recruitment of more ionotropic glutamate receptors.

We saw an enhancement in PPF of CA1 neurons from rats subjected to 30 min of tDCS that persists in the presence of NMDA receptor blocker AP-5. This is consistent with the idea that facilitation is due to the accumulation of calcium in the pre-synaptic cell (Katz and Miledi, 1968; Thomson, 2000), and thereby NMDA receptor independent. However, our data of enhanced PPF contradicts an earlier work in rabbits, in which anodal stimulation induced a decrease in PPF ratio (Marquez-Ruiz et al., 2012). The inconsistency in result is likely due to differences in the brain region that was analyzed as well as the method with which evoked potentials were acquired and measured. We recorded evoked potentials from CA1 neurons in rat ex vivo hippocampal slices, whereas Marquez-Ruiz et al. (2012) recorded from the rabbit somatosensory cortex in vivo.

In contrast to effects on LTP, the tDCS-induced enhancement on PPF can no longer be detected 24 hours following cessation of stimulation. Although there is convincing correlation between anodal tDCS with increased cortical excitability, there is very limited data on whether stimulation can modify pre-synaptic machinery. Our data on PPF provides a glimpse on tDCS effect on the pre-synaptic cell, supporting the hypothesis that neurotransmitter levels at synapses could also be modulated by tDCS.

The unique aspect of our experimental approach is the combination of in vivo treatments with tDCS, followed by extracellular recordings of neurons in freshly prepared hippocampal slices. Changes in synaptic plasticity were observed in these hippocampal slices hours following brain extraction at 30 min and 24 hours following completion of tDCS. For our experiments, brains from both control and stimulated rats were harvested and hippocampal slices were prepared and placed in oxygenated ACSF for 1-6 hours prior to any recording. Therefore, transient electrical field effects of local environment would have dissipated and could not account of observed enhancements in synaptic plasticity. We observed no significant differences in the effects on LTP and PPF on slices recorded in the beginning of the day versus towards the end of the experiment (spanning 4-6 hours). Averaged normalized responses from six CA1 regions within a hippocampal slice from a stimulated rat measured towards the beginning of an experiment showed similar level of potentiation as those from another slice from the same rat measured towards the end of the day (data not shown).

We propose the possibility that the immediate effects of tDCS on local electrical environment induced further downstream signaling events that persists for hours following brain extraction. Cellular changes due to tDCS have been documented previously. An earlier study by Raneiri et al. indicated that in vitro current stimulation of brain slices results in immediate increases in the cFos and zif268, immediate early genes implicated in the maintenance of long term neuronal changes and memory formation (Pérez-Cadahía et al., 2011). However, we cannot rule out the possibility of other faster signaling events such as phosphorylation, recruitment or shuffling of various synaptic proteins in mediating tDCS effects. Experiments in which blockers or

activators are used to block particular signaling cascades (e.g., kinase or phosphatase inhibitors/activators) will be useful in determining the mechanistic pathway of tDCS-induced enhancements in synaptic plasticity.

6.0 CONCLUSIONS

We have demonstrated that in vivo tDCS induces a long lasting enhancement of NMDA dependent synaptic plasticity in the hippocampus of rats. These plastic changes may be the mechanism by which tDCS application facilitates performance in healthy human subjects or alleviates symptoms in patients suffering from neurological disorders. We believe our approach of in vivo tDCS and direct recordings of neuronal signaling in acutely prepared hippocampal slices will continue to yield useful information pertaining to mechanisms of tDCS effects.

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8.0 GLOSSARY

ACSF: artificial cerebrospinal fluid

fEPSPs: field excitatory post-synaptic potentials

LTP: long term potential

PPF: paired pulse facilitation

SEM: standard error of the mean

TBS: theta burst stimulation

tDCS: transcranial Direct Current Stimulation

9.0 APPENDIX

Figure Legends

Figure A-1 (A). Typical positioning of our hippocampal slice in the MED64 probe, containing 64 microelectrodes arranged in an 8x8 array. Dotted rectangular box indicates the hippocampal area that is being recorded whereas the solid rectangular box indicates the typical position where stimulation occurs. (B) Input/output relationship was not affected by tDCS treatment (n = 3 rats, 3 slices). Data are represented as mean±SEM. Sample voltage traces (inset) showing no obvious differences in evoked response between tDCS-treated (red) and control rats (black). Scale bars = 0.4 mV, 10 s. (C) Frequency of spontaneous activity was not significantly different between control and stimulated rats (n=4 rats, 4 slices, P = 0.5). (D) Sample spontaneous spike measurements in a control (top panel) and stimulated (bottom panel) rat. Scale bars = 0.02 mV, 5 sec.

Figure A-2. Effects of tDCS on synaptic plasticity. Rats were subjected to tDCS for 30 min at 250 µA followed by 30 min additional recovery time. (A-D) Effects of tDCS on LTP. (A) Graph of average, normalized slopes of evoked responses from CA1 region of hippocampus from control (Sham, black trace, n=6 rats, 8 slices) or stimulated (tDCS, red trace, 6 rats, 7 slices) rats. Data are presented as means ±SEM. Arrow denotes induction of LTP by theta burst stimulation (TBS). Sample trace of evoked response before (black) and ~30 min after (red) LTP induction by TBS is shown to the right (inset). Scale bar: 0.5 mV, 5 ms. (B) Graph of average, normalized amplitudes of evoked responses from CA1 region of hippocampus from control (Sham, black trace, n=6 rats, 8 slices) or stimulated (tDCS, red trace, 6 rats, 7 slices) rats. Arrow denotes induction of LTP by TBS. Data are presented as means ±SEM. (C,D) Bar graph representing the average percent LTP calculated using slopes (solid fill) and amplitudes (pattern fill) of evoked

responses at 60 min following LTP induction (C) or 30 min following LTP induction (D). Significant enhancements were observed in hippocampal slices from tDCS-treated rats (red) compared to Sham-treated rats (black) (slope data P = 0.002 and 0.01 for 60 and 30 min respectively; amplitude data P = 0.0005 and 0.0002 for 60 and 30 min respectively, df = 13). (E) Effects of tDCS on PPF. PPF ratio was calculated as slope (solid fill) or amplitude (pattern fill) of response due to the 2nd stimulus divided by the respective slope or amplitude of response due to the 1st stimulus. There was a significant increase in PPF ratio in the CA1 region of the hippocampus from rats treated with tDCS (n = 5 rats, 6 slices), compared to that from control (n = 5 rats, 7 slices) (P = 0.003 and 0.005 for slope and amplitude data respectively, df = 65). Data are presented as means ±SEM.

Figure A-3. Effects of decreased tDCS intensity on synaptic plasticity. (A-C) Schaffer collateral-CA1 LTP was significantly enhanced when rats were stimulated with 100 µA for 30 min followed by 30 min recovery time (P < 0.05). (A) A graph of fractional LTP as measured using slopes of field potentials, normalized to baseline and averaged across all animals, showing that rats subjected to tDCS had significantly greater degree of LTP (red) compared to control rats that were subjected to sham (black). Arrow denotes LTP induction by TBS. (B) Bar graph showing significant increases in the average percent LTP resulting from 30 min of 100 µA tDCS (P = 0.01 and 0.04 for slope and amplitude data respectively, df = 10). Percent LTP was calculated using either slopes of fEPSPs (solid fill) or amplitudes of population spikes (pattern fill) recorded in the CA1 region (n = 4 rats, 5 slices). (C) Comparative bar graph indicating some dependence of tDCS-induced LTP enhancement on tDCS intensity. Average slope (solid fill) or amplitude (pattern fill) data from rats subjected to either 250 µA tDCS were normalized against their corresponding Sham data. (E) Rats subjected to 100 µA tDCS displayed significant enhancement

of paired pulse facilitation as measured by slope (solid fill; P = 0.002, df = 42) or amplitude (pattern fill; P = 0.006, df = 42).

Figure A-4. Effects of tDCS on synaptic plasticity assessed in 24 hours. (A,B) LTP was enhanced in rats 24 hours following treatment with tDCS (250 µA, 30 min). (A) Average, normalized slope showing enhanced LTP in tDCS-treated rats (red) compared to sham-treated rats (black). (B) Bar graph of average percent LTP showing significant enhancement of LTP in stimulated rats (red; n=7 rats, 10 slices) compared to control (black; n=7 rats, 8 slices, unpaired 2-tailed t test) as measured using either slope data (solid fill; P = 0.03, df = 16) or amplitude data (pattern fill; P = 0.02, df = 16). (C) Paired pulse facilitation was not significantly altered in rats 24 hours following treatment with tDCS (P = 0.3 – 0.9). Stimuli were set at 30 (black), 40 (red) and 50 (blue) µA. Amplitude measurements were obtained to generate graph. Slope measurements also produced no significant changes in PPF ratio (data not shown). (D) General neurotransmission property were unaltered in rats 24 hours following treatment with tDCS. Average input/output relationship was similar in sham (black) and tDCS rats (red). Sample traces of field potentials evoked by stimuli of varying intensity, as indicated. Vertical scale bar = 0.5 mV. Horizontal scale bar = 5 ms.

Figure A-5. Effects of glutamate receptor blockers on tDCS effects. Rats were subjected to 100 µA for 30 min followed by 30 min recovery time. (A) Measured evoked response mostly mediated by ionotropic glutamate receptors as blockade of kainate, AMPA and NMDA receptors by a cocktail of 30 µM DNQX and 50 µM AP-5 diminished evoked responses from CA1 regions of the hippocampus from both stimulated (red) and control (black) rats. Bar denotes perfusion of DNQX and AP-5. Blockade of NMDA receptor only by AP-5 did not induce measureable changes in field potentials as shown by the sample recording (right inset), showing only a slight

change in response size due to AP-5 perfusion (red) but a dramatic blockade of response due to both AP-5 and DNQX (blue) compared to ACSF only (black) (inset). Scale bar = 10ms, 0.5 mV. (B) Blockade of NMDA receptors by AP-5 diminished LTP in both control (black) and tDCS-treated (red) rats when calculated using fEPSP slope (solid fill) or amplitude (patterned fill). (C) Sample recording from tDCS-treated rat hippocampus showing initial LTP induction (1^{st} arrow) in the presence of normal ACSF, blockade of LTP induction in the presence of ACSF and AP-5 (2^{nd} arrow) and normal LTP induction following wash of AP-5 (3^{rd} arrow). Arrows denote LTP induction by TBS. Red bar indicates perfusion of AP-5. Black bar indicates return to perfusion of normal ACSF. (D) Effect on PPF enhancement due to tDCS were not altered by AP-5 perfusion. In the presence of AP-5, there was still a significant enhancement of PPF (n = 2 rats, 4 slices) in rats subjected to tDCS (red) compared to sham (black) when both slopes (solid fill; $P = 0.02$, $df = 14$) or amplitudes (pattern fill; $P = 0.03$, $df = 14$) values were used for calculation.

Figure A-1

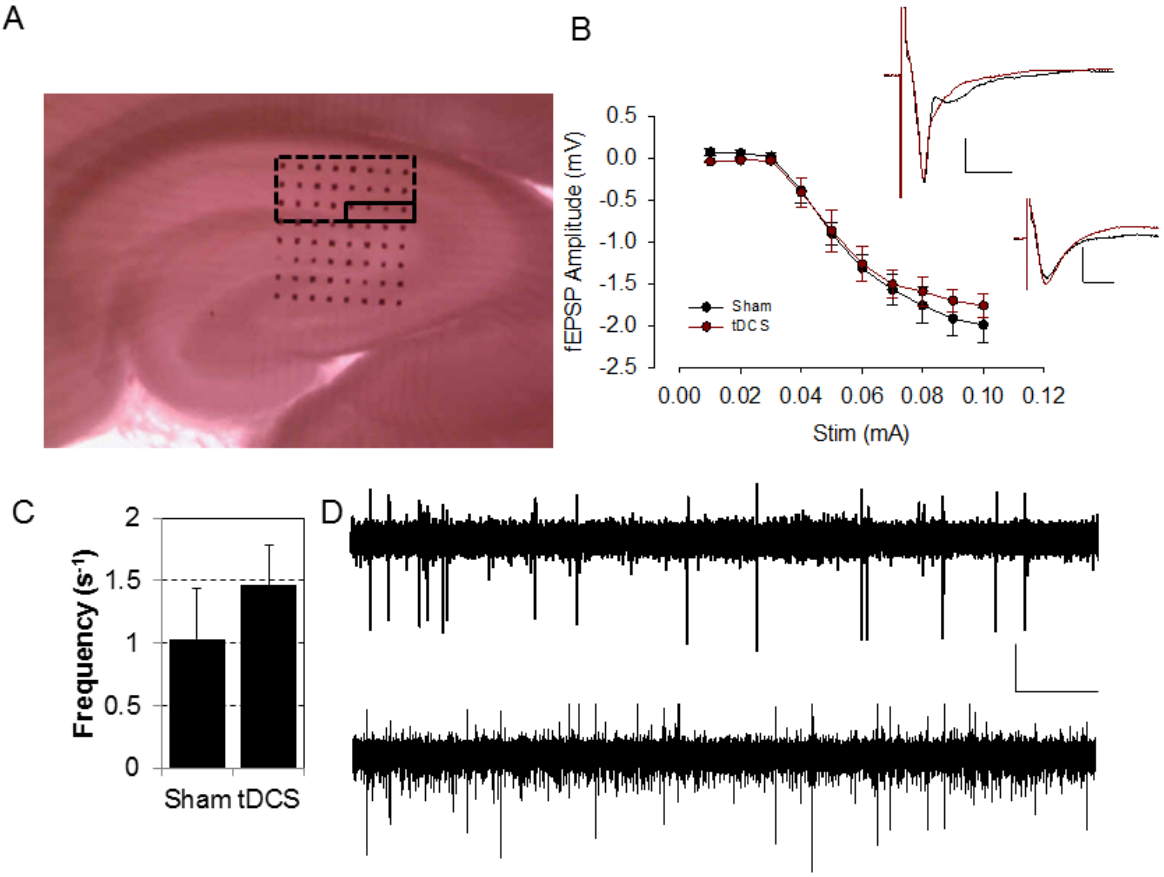

Figure A-2

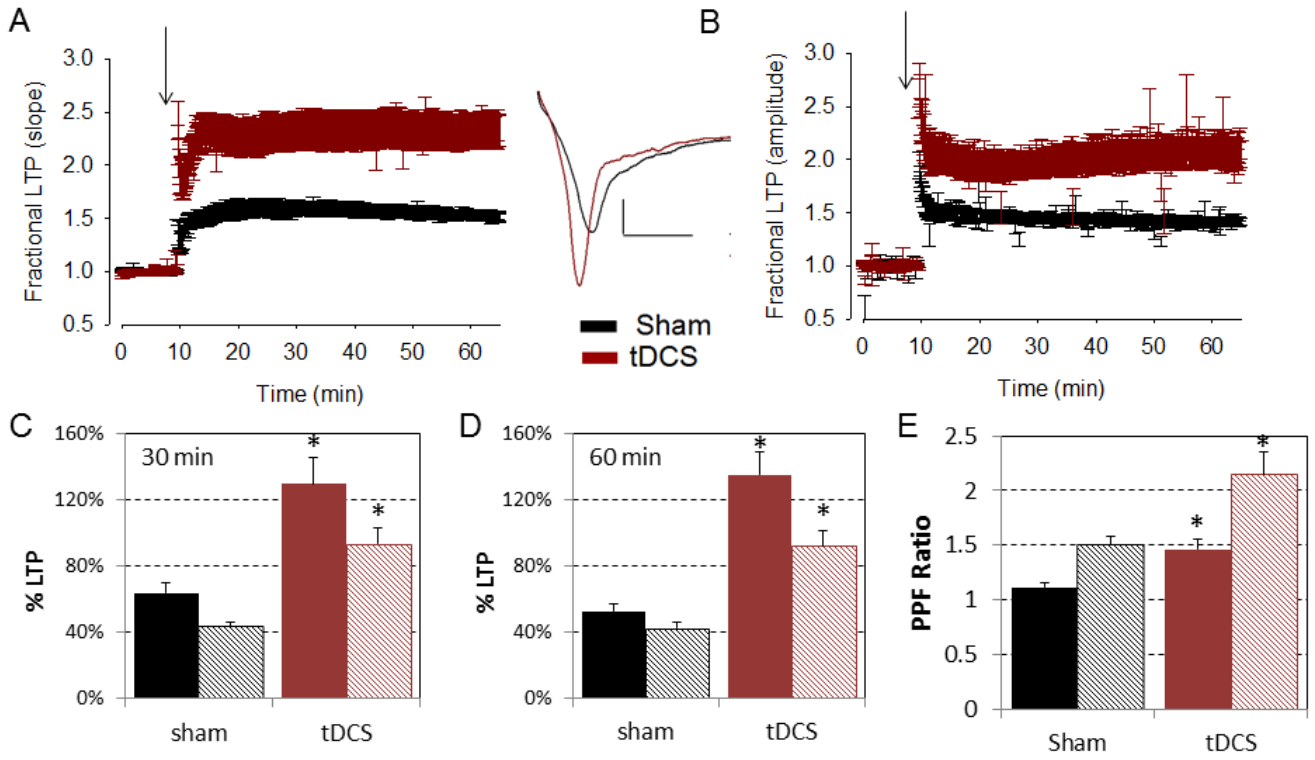

Figure A-3

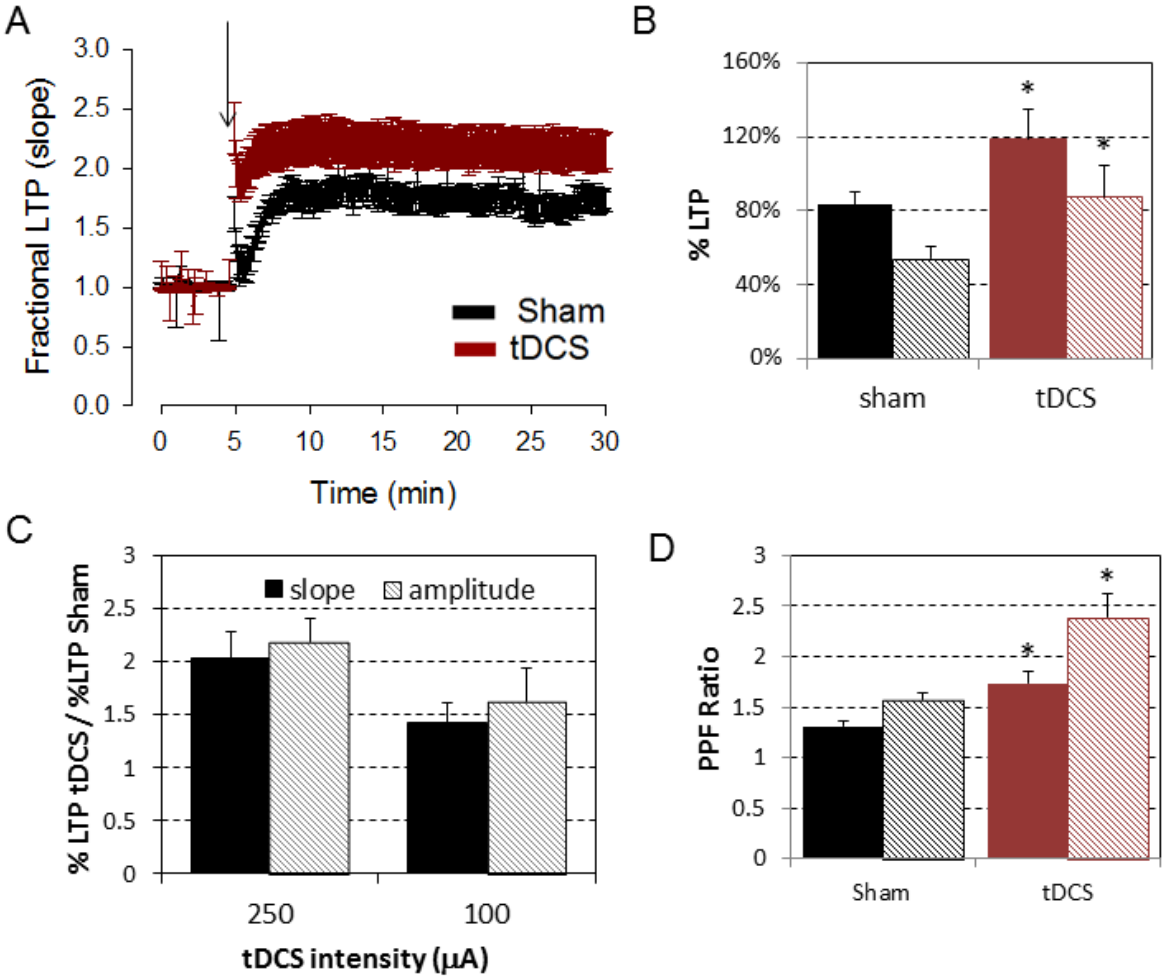

Figure A-4

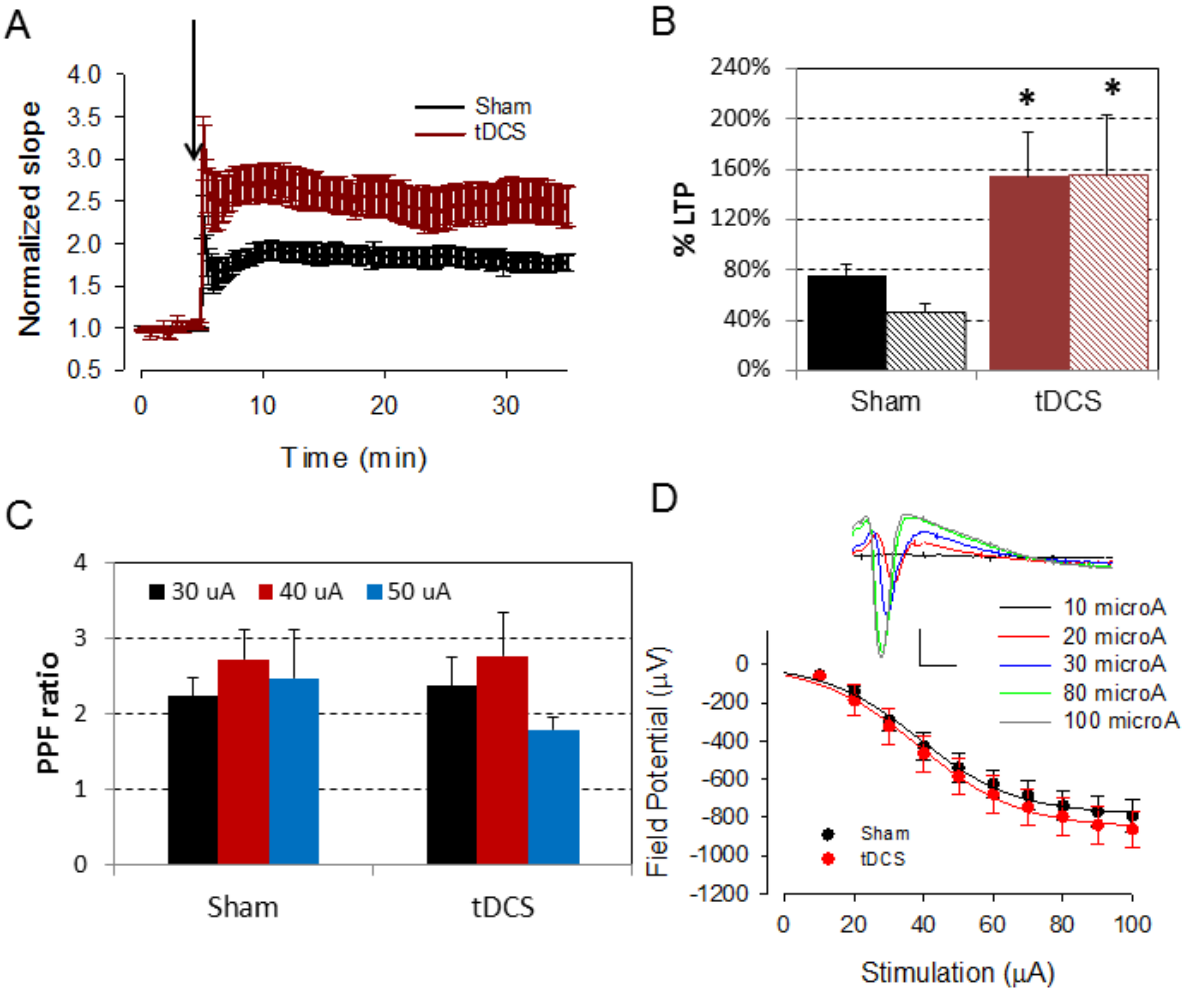

Figure A-5